Quantification of Apolipoprotein A-1 in Cow Serum by Single Radial Immunodiffusion

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ABSTRACT

A single radial immunodiffusion assay was developed to determine the concentration of the major apolipoprotein A-I in bovine serum. Assay was performed using agarose gel plates with monospecific rabbit antiserum. Standards made with bovine high density lipoproteins or serum 10 times diluted were applied in 3-μl amounts to each well in the gel. The assay working range was 2 to 22 mg of apolipoprotein A-I. The intraassay and interassay CV were 3.7 and 4.9%, respectively. Using protein A-I. The intraassay and interassay CY were 3.7 and 4.9%, respectively. Using radial immunodiffusion assay, 23 serum samples from cows at various stages of lactation were analyzed. Apolipoprotein A-I was significantly increased in mid-lactation compared with concentrations in early lactation or dry period.

INTRODUCTION

Plasma lipoproteins are lipid-protein complexes that are heterogenous in composition, size, and biological activity. Although the major lipoprotein fractions are generally evaluated in terms of lipoprotein lipids, apolipoproteins may give additional informations on lipoproteins. The lipoprotein profile of ruminants has been thoroughly reviewed (17, 18). Most circulating lipids are related to the high density lipoproteins (HDL), apolipoprotein A-I (apo A-I)-containing particles. Bovine animals have characteristically low concentrations of triglyceride-rich, apolipoprotein B-containing lipoproteins, but they are of metabolic importance as primary sources of lipids, particularly in the lactating animals. Bovine plasma lipoproteins are usually separated under the same conditions as those used for human plasma lipoproteins. There are no standard conditions for isolating ruminant lipoproteins (14). Isolation of the bovine lipoprotein classes is complicated by the fact that they are present as heterogenous particles. The fraction that corresponds to the low density lipoproteins (LDL) has some features of α- and β-lipoproteins; these latter contain apo A-I particles with HDL-like properties (1, 3, 4, 5, 26). Because of the large distribution of apo A-I containing particles among the lipoproteins and physiological and pathological variations in the amount of HDL-like particles (13, 20), direct determination of apo A-I is needed for bovine lipoproteins. This is the major protein constituent of HDL and can be readily determined by immunological methods. The objective of this report is to describe a simple, yet precise and accurate, single radial immunodiffusion assay (RID) for determination of apo A-I in the bovine serum.

MATERIALS AND METHODS

Purification of Apolipoprotein A-I

Blood was obtained from the jugular vein of a healthy dry cow. Coagulation was prevented with Na_2 EDTA (1 mg/ml blood), and the plasma was separated by low speed centrifugation. High density lipoproteins (d = 1.063 – 1.21 g/ml) were prepared from the plasma by sequential preparative ultracentrifugation (6) in a

Received February 29, 1988.
Accepted September 15, 1988.
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Beckman L8 ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a 50 Ti rotor at 110,000 × *g* and 20°C for 24 h. Before the lipoproteins were separated, NaN_3, merthiolate, and Na_2EDTA were added to provide concentrations in plasma of .02, .005, and .04%, respectively. The isolated HDL was washed by flotation at a density of 1.21 g/ml. After exhaustive dialysis against water and delipidation, protein preparation was solubilized in .01 *M* Tris-HCl, pH 8.3, containing 8 *M* urea, and was eluted with the same buffer by gel filtration using a Sephadex G-150 column (2.6 × 100 cm) (23). Fraction II, corresponding to apo A-I, was collected and 20 μg of the apolipoprotein were electrophoresed on 10% SDS-PAGE (29). The protein concentration of purified apo A-I was determined according to Lowry et al. (10). The gels after electrophoresis were stained with .1% Coomassie brilliant blue R 250 (Sigma Chemical Co., St. Louis, MO) in methanol/acetic acid/H_2O (25:10:65) and destained with methanol/acetic acid/H_2O (10:7.5:82.5). Bands were identified by their apparent molecular weights using protein standards run simultaneously (Pharmacia Fine Chemicals, Uppsala, Sweden). The apo A-I (28,000 daltons) showed a single protein band.

Preparation of Anti-Apolipoprotein A-I Antiserum

Rabbits were injected intracutaneously, three times at 2-wk intervals, with .5 mg of apo A-I emulsified with complete Freund's adjuvant (Miles Laboratories, Elkhart, IN) (vol/vol). Animals were bled 7 d after the last injection. The specificity of the antiserum was studied by Ouchterlony's double diffusion (16), immunoelectrophoresis (24), two-dimensional immunoelectrophoresis, and immunoblot analysis (28) using ^{125}I-labelled conjugate protein A (IM. 144; Amersham, France SA). Antitotal bovine lipoprotein immunoserum as total apolipoprotein binding and nonimmune rabbit serum as control of nonspecific binding were used. Ultracentrifugally isolated lipoprotein fractions, heparin-Sepharose affinity chromatography column purified LDL (30), and bovine serum albumin (fraction V, Sigma Chemical Co.) were utilized for immunoserum purity control.

Apolipoprotein A-I High Density Lipoproteins Standard Preparation

The HDL (d = 1.063 – 1.21 g/ml) was isolated by ultracentrifugation at 110,000 × *g* for 24 h and washed by flotation at a density of 1.21 g/ml. The SDS-PAGE on 10% acrylamide gel and subsequent densitometry (Vernon densitometer) after coloration by Coomassie blue R-250 showed that 89 ± 1% (n = 8) of protein HDL preparation consisted of apo A-I. No corrections for differences in dye uptake between the apolipoproteins were made.

Quantitative Radial Immunodiffusion

The quantification of apo A-I was performed by RID according to Mancini et al. (11) using a 1.25% agarose gel mixed with an accurate amount of anti-apo A-I antiserum. The standard apo A-I HDL at different concentrations and serum diluted 10 times in .15 *M* NaCl were applied in 3-μl amounts to each well in the gel. Plates were incubated for 48 h at 25°C in a humidity chamber followed by washing in .15 *M* NaCl for 24 h. Plates were stained for 15 min in .1% Amido black 10 B in acetic acid (2%) and destained with acetic acid (2%). The ring precipitates were measured by using a projector for immunoanalysis. The effect of dissociation of lipoproteins by heat on the detection of apo A-I was studied in apo A-I HDL standard dilutions and serum (diluted 10 times with .15 *M* NaCl) by heating to 52°C for 3 h (9). Recovery of apo A-I was tested by addition of various concentrations of apo A-I HDL in serum sample.

Serum Sampling

Blood samples were taken from the jugular vein of Holstein × Friesian cows in various physiological stages: dry, late gestation period (n = 7), 5 to 15 d after calving (n = 9), and 6 to 10 mo of lactation (n = 7). Serum was separated from cellular elements at 20°C. Upon separation of serum by low speed centrifugation (2000 × *g*, 20 min), NaN_3, merthiolate, and Na_2EDTA were promptly added to final concentration of .02, .005, and .04%, respectively. Cows were fed ad libitum with a complete diet based on corn silage supplemented with concentrate.

Statistics

Linear regression analysis (25) was used to describe the effect of heat treatment on standard or serum samples. Group means were compared for statistical significance by analysis of variance followed by Student's t test (25) when the former reached significance; $P < .05$ was considered statistically significant.

RESULTS

Immunological Studies

Figure 1a shows the results of Ouchterlony's double immunodiffusion test of antibodies to bovine apo A-I against bovine serum, bovine lipoproteins, and bovine serum albumin. Anti-apo A-I serum formed fused single precipitin line with apo A-I, serum HDL, and 1.006 to 1.063 g/ml density fraction. No precipitin line was formed with albumin. The apo B was the major protein compoent of purified LDL eluted from the heparin-Sepharose affinity chromatography column (unpublished data). No reaction with purified LDL was found.

Immunoelectrophoresis (Figure 1b) and two-dimensional immunoelectrophoresis (Figure 1c) showed that serum and lipoproteins (d = 1.006 to 1.21 g/ml) gave a single precipitin line with the mobility of α-lipoproteins. The immunoblot of the SDS-PAGE using antitotal lipoprotein and anti-apo A-I immunosera is shown in Figure 1d. When anti A-I immunoserum was used, only the band corresponding to the apo A-I was revealed. This indicated that our anti-apo A-I immunoserum was monospecific.

Radial Immunodiffusion Realization

Figure 2 shows the RID plate with apo A-I, HDL standards, and diluted sera. Purified apo A-I gave a ring precipitate with an indistinct edge, whereas HDL standards and sera provided sharp ring precipitates. Heating had no effect on the immunoreactivity of HDL standard (Figure 3) nor on the detection of apo A-I in cow serum samples (Figure 4). The relationship between the squared diameter of the precipitate ring and the concentration of protein apo A-I standard was linear over the range of 2 to 22 mg/dl. The intraassay coefficient of variation for the three serum samples (with low, medium, and high values for apo A-I) measured in replicates (n = 10) on the same gel plate was 3.7% and the interassay coefficient (n = 3) was 4.9%. When serum was enriched in apo A-I with HDL, the apo A-I concentration was proportionally increased (Table 1).

Apolipoprotein A-I in Dairy Cows at Various Physiological Stages

Figure 5 shows serum apo A-I obtained in the dry and the lactation periods. Apo A-I concentrations were significantly increased in midlactation compared with those in early lactation or in the dry period.

DISCUSSION

Apo A-I, the major protein component of the bovine HDL, was isolated and characterized by Jonas (8). This protein was recovered in an essentially pure form in fraction II of the gel filtration. In our work, the use of this preparation gave an anti-apo A-I monospecific im-

TABLE 1. Analytical recovery of apolipoprotein A-I (apo A-I).

Serum	Added apo A-I	Measured apo A-I	Calculated apo A-I	Recovery of apo A-I
	(mg/dl)			(%)
A[1]	...	46	...	...
A + B[1]	69	112	115	97
A + HDL[2]	102	147	148	99
A + HDL	141	183	187	98
A + HDL	174	234	220	106

[1] Twofold diluted cow sera (A, B).

[2] Progressive overcharge of serum with high density lipoproteins (HDL); adjustment for apo A-I content in HDL was made.

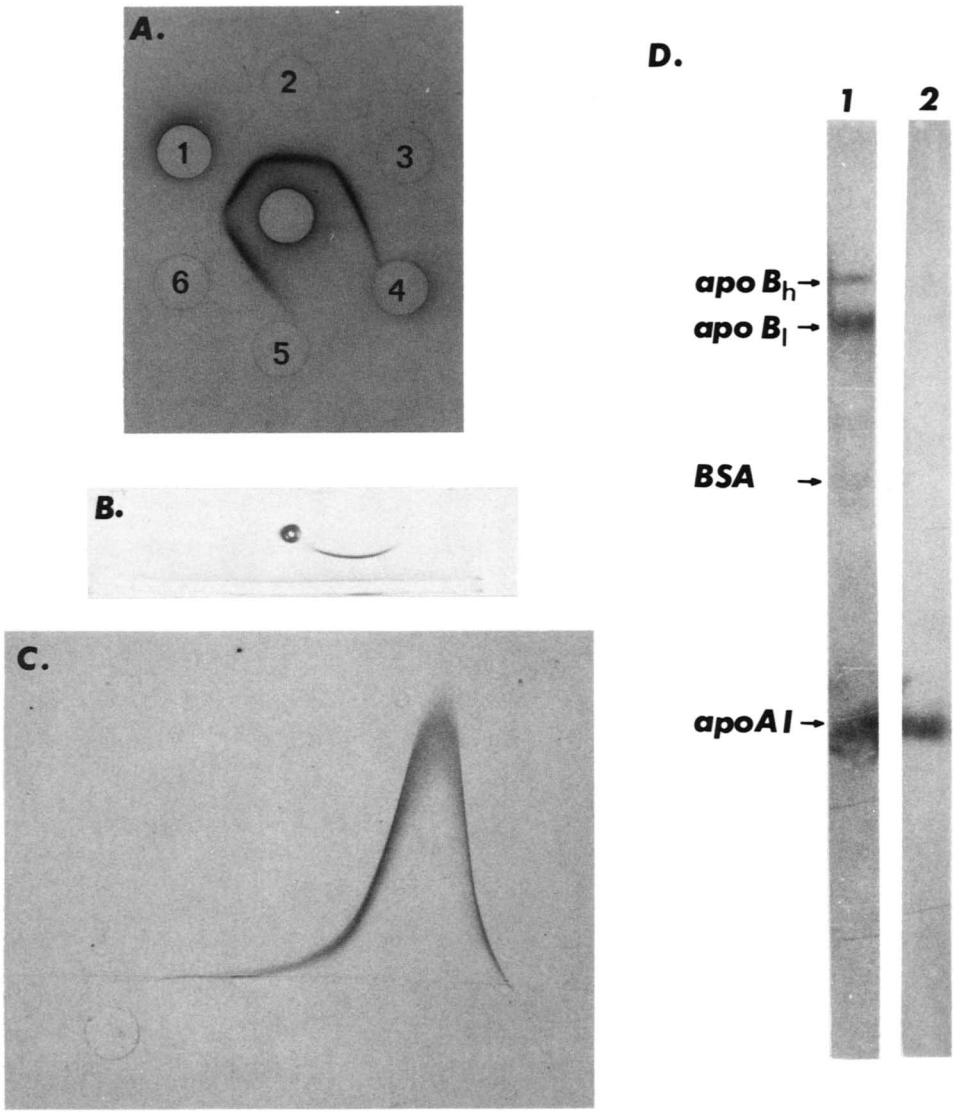

Figure 1. Immunodiffusion and immunoelectrophoresis studies of antiserum to bovine apolipoprotein (apo) A-I prepared in rabbit. A) Immunodiffusion pattern of antiserum (central well, 10 μl) to purified apo A-I (well 1, 10 μg), bovine serum (well 2, 10 μl), HDL (well 3, 10 μg), bovine serum albumin (well 4, 10 μg), purified LDL (well 5, 10 μg), and 1.006 to 1.063 g/ml density fraction (well 6, 15 μg). B) Immunoelectrophoresis, and C) Two-dimensional immunoelectrophoresis of bovine lipoproteins (d = 1.006 to 1.21 mg/ml) against antiserum to apo A-I. D) Autoradiograph of an immunoblot of bovine apolipoproteins (2 μg) electrophoresed on 4 to 20% polyacrylamide gradient gel. Replicas were incubated with antitotal lipoproteins (1) or anti-apo A-I immunoserum (2). Apolipoprotein bands were identified by their apparent molecular weights from calibration of the gel using protein standards.

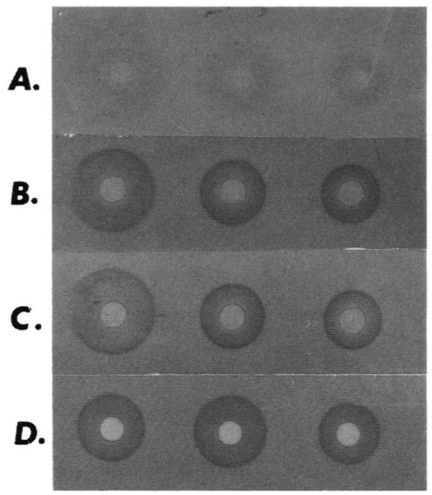

Figure 2. Radial immunodiffusion assay with the agarose gel plate containing rabbit anti-cow apolipoprotein (apo) A-I antiserum. A) Purified apo A-I (20, 15, 10 μg). B) Untreated HDL apo A-I standard (20, 15, 10 μg of apo A-I, adjustment for apo A-I content in HDL was made). C) The same HDL treated by heat at 52°C for 3 h. D) Representative cow serum samples (diluted 10-fold with saline, 3 μl per well).

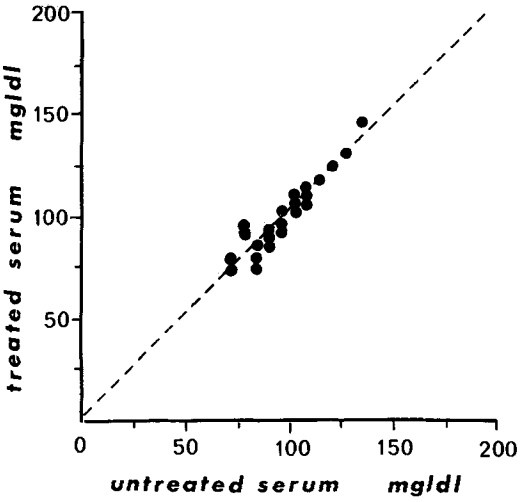

Figure 4. Relationship between the radial immunodiffusion assay realized with untreated and heat treated cow serum (n = 23, Y = 3.118 + 1.017, r = .936).

munoserum that did not react with the most contaminating proteins like albumin or purified LDL.

Apolipoproteins in the serum are present as a part of the lipid-protein complexes. A major difficulty encountered in the immunoassay for apo A-I has been the inability to detect the full complement of apo A-I in the native lipoprotein, because some protein immunoreactive sites were masked by the lipid portion. Methodological problems and aspects of standardization of apo A-I immunoassays in human serum have recently been reviewed (27). The choice of

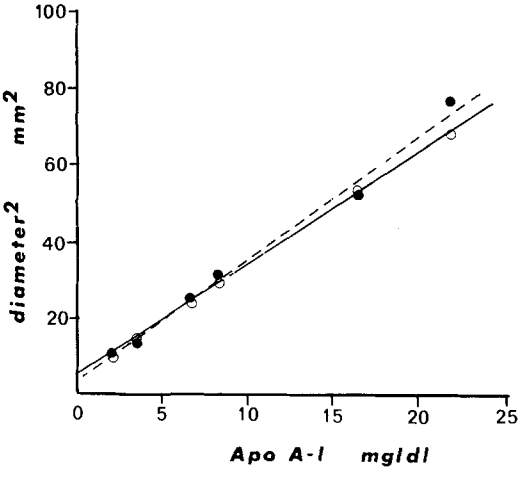

Figure 3. Relationship between precipitate ring size (diameter^2) to apo A-I concentration in untreated (—○—, Y = 5.644 + 2.878x; r = .998) and heat-treated HDL standard (—●—, Y = 3.637 + 3.263x; r = .995).

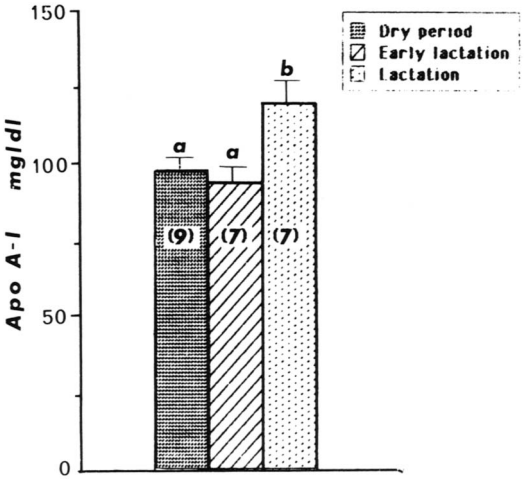

Figure 5. Apolipoprotein (apo) A-I values in cow serum at various physiological stages. ^{ab}Means with different letters differ ($P<.05$).

standard preparation and treatment of samples for immunoanalysis still remain questionable.

The immunological reactivity of delipidated apo A-I may differ from the apo A-I in HDL because of modifications in protein conformation during the purification step of the apoprotein. This difference might seriously affect apo A-I determination using purified apo A-I as a standard. Our work has shown that it is possible to use isolated HDL as a standard according to results obtained in human serum (7). The use of this procedure also eliminates the need for purified apo A-I in the assay standard preparation.

Treatment of samples (delipidation, dissociation) is frequently necessary for full detection of the apo A-I by immunochemical methods. Karlin et al. (9) proposed heating of the samples for maximal exposition of the apo A-I antigenic sites without altering the apo A-I antigenicity. Heating presumably changes the interaction between apo A-I and lipids and renders the antigenic sites accessible to the antibody. In our study no detectable change in reactivity of the HDL and serum was observed after heat treatment. In the human serum, various responses on immunodetection of apo A-I were obtained after heating (9, 13, 21, 22). Probably most of the variability lies in the antiserum used. The amount of apo A-I is likely to depend on the titer of those antigenic sites exposed in the native HDL. Use of the surface specific antibodies to determine the apo A-I concentration in samples without prior physical or chemical treatment must be considered in the future (2, 12).

Determination of apo A-I in cows at various stages of lactation indicated that concentrations were increased in midlactation compared with early lactation or the dry period. This result agrees with evolution of lipids and lipoproteins studied previously in dairy cows (13, 19). The apo A-I is the major apolipoprotein of cow lipoproteins; therefore, variations in the apo A-I concentrations are related to a large extent with variations in serum lipoproteins.

In conclusion, similar to results obtained with human serum (27), RID provides a simple but reliable method for determining apo A-I in bovine serum.

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